

## Mutation in *cyaA* in *Enterobacter cloacae* decreases cucumber root colonization

Daniel P. Roberts · Laurie F. McKenna · Xiaojia Hu ·  
Scott M. Lohrke · Hye Suk Kong · Jorge T. de Souza ·  
C. Jacyn Baker · John Lydon

Received: 21 June 2006 / Revised: 29 August 2006 / Accepted: 1 September 2006 / Published online: 6 October 2006  
© Springer-Verlag 2006

**Abstract** Strains of *Enterobacter cloacae* show promise as biological control agents for *Pythium ultimum*-induced damping-off on cucumber and other crops. *Enterobacter cloacae* M59 is a mini-Tn5 Km transposon mutant of strain 501R3. Populations of M59 were significantly lower on cucumber roots and decreased much more rapidly than those of strain 501R3 with increasing distance from the soil line. Strain M59 was decreased or deficient in growth and chemotaxis on most individual compounds detected in cucumber root exudate and on a synthetic cucumber root exudate medium. Strain M59 was also slightly less acid resistant than strain 501R3. Molecular characterization of strain

M59 demonstrated that mini-Tn5 Km was inserted in *cyaA*, which encodes adenylate cyclase. Adenylate cyclase catalyzes the formation of cAMP and cAMP levels in cell lysates from strain M59 were approximately 2% those of strain 501R3. Addition of exogenous, nonphysiological concentrations of cAMP to strain M59 restored growth (1 mM) and chemotaxis (5 mM) on synthetic cucumber root exudate and increased cucumber seedling colonization (5 mM) by this strain without serving as a source of reduced carbon, nitrogen, or phosphorous. These results demonstrate a role for *cyaA* in colonization of cucumber roots by *Enterobacter cloacae*.

D. P. Roberts (✉) · L. F. McKenna · S. M. Lohrke ·  
H. S. Kong · J. Lydon  
Sustainable Agricultural Systems Laboratory,  
USDA-Agricultural Research Service,  
Bldg. 001, Rm. 140, 10300 Baltimore Avenue,  
Beltsville, MD 20705-2350, USA  
e-mail: robertsd@ba.ars.usda.gov

X. Hu  
Oil Crops Research Institute,  
Chinese Academy of Agricultural Sciences,  
Baojian, Wuchang, Wuhan 430062,  
People's Republic of China

J. T. de Souza  
Wye Research and Education Center,  
University of Maryland,  
Queenstown, MD 21658, USA

C. J. Baker  
Molecular Plant Pathology Laboratory,  
USDA – Agricultural Research Service,  
Bldg. 004, 10300 Baltimore Avenue,  
Beltsville, MD 20705, USA

*Present Address:*  
S. M. Lohrke  
Naval Health Research Center,  
Environmental Health Effects Laboratory,  
Geo-Centers, Inc.,  
Wright-Patterson Air Force Base,  
OH 45433, USA

*Present Address:*  
H. S. Kong  
Laboratory of Methods Development,  
CBER, FDA, Rockville,  
MD 20852, USA

*Present Address:*  
J. T. de Souza  
CEPLAC/CEPEC/SEFIT,  
Caixa Postal 7, Km 22, Rodovia Ilheus-Itabuna,  
BA, Brazil

**Keywords** Biological control · Colonization · Cyclic AMP · *cyaA* · Rhizosphere

## Introduction

Beneficial bacteria have been applied to soil in association with plant parts for applications including biological control, plant-growth promotion, and bioremediation (Bloemberg and Lugtenberg 2001; Kuiper et al. 2004). There is compelling evidence that root colonization plays an essential role in the successful application of these beneficial bacteria in certain situations (Bull et al. 1991; Chin-A-Wong et al. 2000). Traits involved in colonization of subterranean plant parts include chemotaxis, putrescine uptake, prototrophy, stress tolerance, and the production of antibiotics, NADH dehydrogenases, site-specific recombinase, and certain bacterial surface molecules (Mazzola et al. 1992; Rainey 1999; Lugtenberg et al. 2001; de Weert et al. 2002; Lohrke et al. 2002; Martinez-Granero et al. 2005).

Some work has been performed regarding the importance of regulatory pathways in plant-beneficial bacteria to colonization of roots by these microbes (Natsch et al. 1994; Chancey et al. 2002). A functional *gacA/gacS* two component regulatory cascade was shown to be important for competitiveness of certain pseudomonads in plant rhizosphere in experiments conducted in natural soil but not in experiments conducted in sterile soil (Chancey et al. 2002). The importance of the *gacA/gacS* system in enhancing competitiveness of pseudomonads in natural soils was attributed to its role in the production of a number of different antibiotics such as phloroglucinols, phenazines, pyrrolnitrin, lipopeptides, and hydrogen cyanide (Heeb and Haas 2001). Many of these compounds have been shown to contribute to rhizosphere competence (Mazzola et al. 1992; Natsch et al. 1994; Whistler et al. 1998; Bull et al. 2001). A possible two component regulatory system with homology to OmpR–PhoB response regulators has also been shown to be important for rhizosphere competence (Dekkers et al. 1998).

Cyclic AMP (cAMP) is a regulatory molecule widely distributed among prokaryotes and eukaryotes (Botsford and Harman 1992; Tang and Gilman 1992). In *Escherichia coli* and other enteric bacteria cAMP regulates gene expression when bound to the cAMP-receptor protein (CRP). The cAMP–CRP complex is well known for regulating the acquisition and utilization of carbon in bacteria and is also involved in regulation of flagellar production and responses to heat, pH, and osmotic stresses (Botsford and Harman 1992; Ferenci 1999; Kimura et al. 2002; Ma et al. 2003). Additionally,

the cAMP–CRP complex has been shown to interact with other transcriptional regulators, such as Fur, which regulates iron utilization (Zhang et al. 2005). Functional interactions between nutritional regulons are thought to be important in balancing the supply of essential nutrients to the bacterial cell (Gutierrez-Rios et al. 2003). The balanced acquisition of nutrients and overcoming these environmental stresses may have a positive influence on root colonization implicating a role for the cAMP–CRP complex in root colonization. However, there have been no studies to our knowledge analyzing the role of cAMP or adenylate cyclase, the enzyme that catalyzes the synthesis of cAMP, in colonization by plant-beneficial bacteria.

Strains of the enteric, plant-beneficial bacterium *Enterobacter cloacae* have been shown to suppress *Pythium ultimum* damping-off of cucumber, cotton, and other crops (Hadar et al. 1983; Nelson 1988). *Enterobacter cloacae* also colonizes the subterranean portions of a number of plant species (Nelson 1988; Roberts et al. 1996, 1999; Lohrke et al. 2002). *Enterobacter cloacae* M59 is a prototrophic, transposon mutant of *Enterobacter cloacae* 501R3 that was shown to be deficient in growth on several amino acids and in colonization of seeds of cucumber and other crops (Roberts et al. 1996). We characterized the mutation in strain M59 at the molecular level in an attempt to understand the colonization behavior of this strain. We demonstrate here that strain M59 contains a mutation in *cyaA*, which encodes adenylate cyclase, and that *cyaA* is important for colonization of cucumber roots by *Enterobacter cloacae*.

## Materials and methods

### Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. *Enterobacter cloacae* 501R3 is a prototrophic, spontaneous, rifampicin-resistant mutant of *Enterobacter cloacae* EcCT501 (Nelson 1988; Roberts et al. 1992). *Enterobacter cloacae* M59 is a mini-Tn5 Km mutant of 501R3 (Roberts et al. 1996). When appropriate the growth medium used to culture these strains was supplemented with ampicillin (Ap, 50 µg ml<sup>−1</sup>), kanamycin (Kan, 50 µg ml<sup>−1</sup>), or rifampicin (Rif, 100 µg ml<sup>−1</sup>).

### Molecular techniques

DNA isolations, transformations, electroporations, restriction enzyme digestions, electrophoresis, and

**Table 1** Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
<b>Strains</b>		
<i>E. cloacae</i> 501R3	Spontaneous Rif <sup>r</sup> mutant of <i>E. cloacae</i> EcCT501	Roberts et al. (1992)
<i>E. cloacae</i> M59	Mutant of <i>E. cloacae</i> 501R3 containing <i>cyaA</i> ::mini-Tn5 Km; Kan <sup>r</sup> , Rif <sup>r</sup>	Roberts et al. (1996), this study
<i>E. coli</i> DH5 $\alpha$	(80 <i>dlacZ</i> ΔM15)( <i>lacZYA-argF</i> ) U169 <i>glnV44 deoR gyrA96 recA1 relA91 endA1 thi-1 hsdR17</i>	Sambrook and Russell (2001)
<b>Plasmids</b>		
pM59	Kan <sup>r</sup> , Ap <sup>r</sup> , contains 6.0-kb <i>ApaI</i> -fragment from <i>Enterobacter cloacae</i> M59 containing <i>cyaA</i> ::mini-Tn5 Km	This study
pGEM-7Zf(+)	Ap <sup>r</sup> , cloning vector	Promega Corp.

Ap<sup>r</sup> ampicillin resistant, Kan<sup>r</sup> kanamycin resistant, Rif<sup>r</sup> rifampicin resistant

ligations were performed as previously described (Sambrook and Russell 2001). Plasmid pM59 (Table 1) was constructed by ligating *ApaI*-digested M59 genomic DNA to *ApaI*-digested pGEM-7Z(+) followed by electroporation into *E. coli* DH5 $\alpha$  and isolation of Ap- and Kan-resistant colonies. The nucleotide sequence of both strands of the *Enterobacter cloacae* portion of pM59 was obtained using PCR-mediated Taq DyeDeoxy terminator cycle sequencing. Primers designed to the right (MT5R2096: 5'-GGG CCT TGA TGT TAC CGA GAG C-3') and left (MT5L294: 5'-TAA GCG TGC ATA ATA AGC CCT ACA-3') ends of mini-Tn5 Km (Lohrke et al. 2002) were used to sequence out from this transposon in pM59. The upstream portion of *cyaA* and adjacent sequence was obtained by directional walking (Mishra et al. 2002). Primers AMPBTF2: 5'-Bio-AAT CGG CAG TTC ACC TTT TGG AGA-3' (Bio-biotin) and AMPPF2: 5'-AGG TAA TCG TGT TGG GTT TCA-3' were used in the first walking experiment and primers AMPBT3: 5'-Bio-CGC TTC ATC AAC CGT AAA ACT G-3' and AMP3: 5'-TGT TAG GAT GGT TAG CGA TTG ATA-3' in the second. PCR amplifications were done as described by Mishra et al. (2002). Amplified products were excised from the gel, purified with GeneClean (MP Biomedicals Co., Solon, OH, USA) using conditions recommended by the supplier, and sequenced by PCR-mediated Taq DyeDeoxy terminator cycle sequencing. The LASERGENE (DNASTAR, Inc., Madison, WI, USA) sequence analysis software package and BLAST searches (Altschul et al. 1997) were used for DNA and protein sequence analyses. Multiple sequence alignments were performed with CLUSTAL W version 1.81 (Thompson et al. 1994). The GeneContext analysis was performed with the Gene Context Tool (Ciria et al. 2004) on 8/15/2006 with 345 microbial genomes included.

For Southern hybridization, genomic DNA was isolated, digested, separated, and hybridized using standard protocols (Sambrook and Russell 2001). Probes for mini-Tn5 Km and *cyaA* were constructed by PCR. PCR amplifications were performed in 25  $\mu$ l reactions with reaction mixtures consisting of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 10 pMole primer; 200  $\mu$ Mole each dNTP; 1 U AmpliTaq Gold (Perkin-Elmer); and 40–100 ng of bacterial DNA. *Enterobacter cloacae* 501R3 and M59 genomic DNA were used as template for construction of *cyaA* and mini-Tn5 Km probes, respectively. Primers for PCR were Tn5F1: 5'AAC GCA AGC GCA AAG AGA AAG AGG TAG-3', Tn5R1:5'-TCA TAG AAG GCG GCG GTG GAA TC-3', *cyaA*F1:5'-CTG TGC GTC TGG CCG GGA AGC GTA TT-3', and *cyaA*R1:5'-TGC CGT CGG GTC GTA TTC CAG GTT GA-3'. For PCR construction of the mini-Tn5 Km probe, denaturation was at 95°C for 10 min for the first cycle and 94°C for 30 s for each subsequent cycle. Annealing and elongation were at 58°C for 20 s and 72°C for 30 s, respectively, for 5 cycles with 2 s added to the annealing time per cycle. Annealing and elongation for an additional 25 cycles were at 58 and 72°C for 30 s with 1 s added to the annealing and elongation time per cycle. Reactions were terminated after a final 5 min elongation at 72°C. PCR was as above for construction of the *cyaA* probe except the annealing temperature was 60°C. The amplicons in the reaction mix were separated by electrophoresis and extracted from gels with GeneClean (MP Biomedicals Co.) using conditions recommended by the supplier. Probes were labeled with random primer biotinylation reactions using the NEBlot Photoprobe Labeling Kit according to the manufacturer's instructions (New England Biolabs, Ipswich, MA, USA). Southern hybridizations were conducted at 68°C overnight in 6 $\times$  SSC. Southern blots were developed according to the manufacturer's instructions (New England Biolabs).

### Nucleotide sequence accession number

The nucleotide sequence and sequence of the translated proteins have been deposited in GenBank under accession number AY765258.

### Determination of cAMP levels in cells

*Enterobacter cloacae* M59 and 501R3 were grown overnight to stationary phase in M56 salts (Nguyen et al. 1983) broth plus 0.2% glycerol and 250 rpm at 28 or 37°C. Cells from overnight cultures were collected by centrifugation, washed twice with sterile distilled water (SDW), and resuspended in SDW to O.D. 540 nm = 1.50. Cells (10 ml) in this suspension were centrifuged, resuspended in 2 ml SDW, and lysed with CellLytic Express (Sigma Chemical Co., St Louis, MO, USA) using conditions specified by the manufacturer. cAMP in the lysate was quantified with a competitive enzyme immunoassay (Cat. No. CA-201, Sigma Chemical Co.) using conditions specified by the manufacturer. Total protein in the lysate was determined with the Bradford assay (BioRad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard. The experiment was performed at least 3 times with each strain grown at 28 and at 37°C. Mean pMole cAMP ( $\mu\text{g protein}^{-1}$ ) with standard deviation was determined for each strain. Results from separate experiments were combined prior to analysis.

### Phosphomycin resistance assays

Phosphomycin resistance with strains 501R3 and M59 in the presence and absence of cAMP was determined essentially as described (Sakamoto et al. 2003). Strains were grown overnight in nutrient (N) broth with or without 1 mM cAMP, washed in SDW, resuspended in SDW to O.D. 540 nm = 1.00, and spread on N agar plates with or without 1 mM cAMP. Filter disks containing 25  $\mu\text{l}$  of 0, 500, 1,000, 1,500, 2,000, 4,000, or 6,000  $\mu\text{g ml}^{-1}$  phosphomycin were applied to the plates and the plates incubated 48 h at 37°C prior to examination for zones of inhibition. The experiment was performed twice with three replicates per treatment and experiments analyzed independently.

### Environmental stress assays

Heat stress experiments were performed essentially as described (Delaney 1990). Strains 501R3 and M59, cultured overnight in M56 salts broth plus 0.2% glycerol at 37°C and 250 rpm, were washed twice in M56 salts broth without a carbon source and resuspended to

approximately  $1 \times 10^9$  CFU  $\text{ml}^{-1}$  in M56 salts broth without carbon. Suspensions (330  $\mu\text{l}$ ) of each strain were pipetted into sterile glass test tubes (1.2-cm diameter  $\times$  7.5 cm), incubated at 50°C in a water bath, and CFU  $\text{ml}^{-1}$  determined after 0, 15, 30, 45, 60, 75, and 90 min by dilution-plating onto Luria–Bertani (LB; Sambrook and Russell 2001) agar containing the appropriate antibiotics. Mean  $\text{Log}_{10}\text{CFU}$  with standard deviation was calculated for each strain at each time. The experiment was performed twice with three replicates per treatment and experiments analyzed independently.

Acid resistance with strains 501R3 and M59 was determined as described (Castanie-Cornet et al. 1999). Strains were grown overnight in LB broth plus 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5; diluted 1:1,000 in EG broth, pH 2.5 (Vogel and Bonner 1956) or LB broth plus 100 mM MES, pH 7.0; and incubated at 37°C prior to plating at various times onto LB agar containing appropriate antibiotics. Experiments were performed four times with three replicates per treatment and experiments analyzed independently.

Osmosensitivity with strains 501R3 and M59 was determined on N agar or on N agar supplemented with 1.0, 2.0, or 3.0 M sorbitol; 1.0, 2.0, or 3.0 M glucose; 0.5, 1.5, or 1.5 M NaCl; or 0.5, 1.0 M, or 1.5 M KCl essentially as described (Aiba et al. 1998). Strains were grown overnight at 37°C in N broth, washed in SDW, resuspended in SDW to O.D. 540 nm = 1.00 and 5  $\mu\text{l}$  of this suspension spotted onto the above media and streaked. Agar plates were incubated at 37°C and examined for growth after 48 h. Experiments were performed three times with two replicates per treatment and experiments analyzed independently.

### In vitro growth assays

To determine relative growth on various carbon sources supplied individually, *E. cloacae* strains cultured overnight in M56 salts broth + 0.2% glycerol were harvested by centrifugation, washed twice in 10 mM  $\text{MgSO}_4$ , resuspended in 10 mM  $\text{MgSO}_4$  to O.D. 540 nm = 1.00, and 100  $\mu\text{l}$  of this suspension added to 5 ml M56 basal salts + 0.2% test carbon source in sterile test tubes (1.8-cm diameter  $\times$  15 cm). Carbon sources supplied individually are listed in Table 2. Relative growth on synthetic cucumber root exudate was performed as above using a mixture of reduced carbon compounds detected in cucumber root exudate (D.P. Roberts, L. McKenna, J. Buyer, unpublished results). A stock solution containing (per liter) 0.007 g *L*-arabinose, 0.014 g cellibiose, 0.004 g fructose, 0.014 g galactose, 0.006 g gentiobiose, 0.038 g glucose, 0.002 g

**Table 2** In vitro growth and swarm response by *Enterobacter cloacae* strains on predominant carbohydrates, amino acids, and organic acids in cucumber root exudate

Compound <sup>a</sup>	In vitro growth by M59 <sup>b</sup>	Swarm response (mm) <sup>c</sup>	
		501R3	M59
Carbohydrate			
Arabinose	S	0.0 ± 0.0	0.0 ± 0.0
Cellobiose	S	22.0 ± 0.0	17.3 ± 0.5 <sup>d</sup>
Galactose	S	29.0 ± 0.8 <sup>e</sup>	24.0 ± 2.2 <sup>e</sup>
Glucose	S	37.5 ± 2.5 <sup>e</sup>	27.7 ± 1.7 <sup>e</sup>
Mannose	S	19.0 ± 0.0	7.3 ± 1.2
Rhamnose	S	0.0 ± 0.0	0.0 ± 0.0
Sucrose	WT	22.0 ± 0.0	19.0 ± 0.8
Xylose	S	17.8 ± 0.5	8.0 ± 0.0
Amino acid <sup>f</sup>			
L-Aspartate	S	7.5 ± 0.0	0.0 ± 0.0
L-Glutamine	S	14.3 ± 0.0	0.0 ± 0.0
L-Histidine	S	0.0 ± 0.0	0.0 ± 0.0
L-Proline	NG	17.0 ± 0.0	0.0 ± 0.0 <sup>g</sup>
L-Serine	S	0.0 ± 0.0	0.0 ± 0.0
Organic acid			
Citrate	S	29.7 ± 0.9	5.0 ± 0.0 <sup>d</sup>
Malate	S	30.7 ± 3.1	13.3 ± 1.7
Succinate	S	12.3 ± 0.5	0.0 ± 0.0

<sup>a</sup> Carbohydrate, amino acid, and organic acid compounds tested were present at  $\geq 1 \mu\text{g plant}^{-1}$  in cucumber root exudate (D.P. Roberts, L. McKenna, J.S. Buyer, unpublished). Only compounds that supported growth by strain 501R3 were tested

<sup>b</sup> WT wild-type growth, growth by strains M59 and 501R3 was similar. S slow growth, there was significant growth by strain M59 but the O.D. 540 nm of strain M59 was more than one standard deviation less than that of strain 501R3 at one or more time points. NG no growth, O.D. 540 nm was less than 0.15 at the end of the 8 h experiment. Results from a single experiment are presented

<sup>c</sup> Distance in millimeters migrated toward the reduced carbon compound was determined visually after 48, 72, and 96 h. Except where indicated otherwise, 72 h determinations are reported. Results from a single experiment are presented

<sup>d</sup> Distance migrated by strain M59 was similar to that of strain 501R3 in one of four experiments

<sup>e</sup> Distance migrated was determined after 48 h

<sup>f</sup> In vitro growth data on amino acids is from Roberts et al. (1996). A single experiment with these amino acids was performed for verification. Results from this experiment were consistent with those published previously (Roberts et al. 1996)

<sup>g</sup> Swarm response by strain M59 was apparent in three of three experiments after 96 h. In all experiments, distance migrated by strain M59 was significantly less than that by strain 501R3

isomaltose, 0.004 g lactose, 0.003 g maltose, 0.005 g mannitol, 0.017 g rhamnose, 0.085 g sucrose, 0.006 g trehalose, 0.009 g xylose, 0.092 g citrate, 0.335 g  $\alpha$ -ketoglutarate, 0.032 g malate, 0.604 g succinate, 0.037 g arginine, 0.477 g aspartate, 0.007 g  $\gamma$ -aminobutyric acid, 0.094 g glutamine, 0.022 g glycine, 0.109 g histidine, 0.183 g isoleucine, 0.869 g leucine, 0.020 g lysine, 0.034 g methionine, 0.004 g ornithine, 0.100 g phenylalanine, 1.362 g proline, 0.296 g serine, 0.004 g threonine,

0.035 g tryptophan, and 0.009 g tyrosine was prepared and added to M56 salts broth so that the final concentration of reduced carbon was 0.05, 0.1, or 0.5%. Sugars were the D stereoisomer and amino acids were the L stereoisomer unless indicated otherwise. To determine relative growth on certain reduced carbon sources in the presence of cAMP, experiments were conducted as above except treatments contained 1 mM cAMP. Treatments were incubated at 37°C and 250 rpm. Experiments were performed at least three times with three replicates per treatment and experiments analyzed independently. Growth was monitored by determining O.D. 540 nm. The following rating system was used to compare in vitro growth by strains 501R3 and M59: WT, wild-type growth, growth by strains M59 and 501R3 was similar. S, slow growth, there was significant growth by strain M59 but the O.D. 540 nm of strain M59 was more than one standard deviation less than that of strain 501R3 at one or more time points. NG, no growth, O.D. 540 nm was less than 0.15 after 8 h.

For experiments determining if *E. cloacae* could grow on cAMP as a source of reduced carbon, nitrogen, or phosphorous, strains were grown overnight as above, washed with M56 salts broth without carbon, nitrogen, or phosphorous, respectively, and resuspended in the appropriate M56 salts solution to O.D. 540 nm = 1.00. This suspension (500  $\mu\text{l}$ ) was added to 20 ml test treatment in 250 ml Erlenmeyer flasks. For determining growth on cAMP as a reduced carbon source, treatments were M56 salts broth plus: 0.5% fructose, 5 mM cAMP, and no carbon source. For determining growth on cAMP as a nitrogen source, treatments were M56 salts broth without nitrogen plus: 0.5% fructose, 5 mM L-alanine + 0.5% fructose, and 5 mM cAMP + 0.5% fructose. For determining growth on cAMP as a phosphorous source, treatments consisted of M56 salts broth without phosphorous plus: 0.5% fructose, 5 mM  $\text{KPO}_4$  + 0.5% fructose, and 5 mM cAMP + 0.5% fructose. Flasks were incubated at 37°C and 250 rpm. Growth was measured by determining O.D. 540 nm. Treatments were replicated three times and all experiments performed twice. Experiments were analyzed independently.

### Motility assays

Strains were grown overnight in Kaiser and Hogness salts buffer (Kaiser and Hogness 1960) plus 0.1% glycerol at 30°C and 60 rpm and evaluated for motility microscopically and in open tubes embedded in semi-solid agar (Kreig and Gerhardt 1981) at room temperature. All experiments were performed twice with three replicates per strain.

### Swarm assay

Strains were tested for swarm response toward individual reduced carbon compounds essentially as described (Hawes et al. 1988). Strains were grown overnight as for motility assays with or without 5 mM cAMP, washed twice with chemotaxis wash buffer (Adler 1973), and resuspended in chemotaxis buffer (Adler 1973) so that bacterial suspensions contained approximately  $10^{10}$  CFU ml<sup>-1</sup>. Bacterial suspensions (10 µl) were spotted at the center of a Petri dish (100-mm diameter) containing swarm medium (Hawes et al. 1988) with or without 0.3 mM cAMP. The test carbon source (10 µl, 100 mM) was spotted 3 cm from the bacterial suspension at the periphery of the plate and the plates incubated at 28°C without shaking. Carbon sources applied individually are listed in Table 2. Swarm response toward 0.4% synthetic cucumber root exudate medium (described above) was also tested. For these experiments, swarm agar was prepared with and without 0.3 mM cAMP. Plates were inspected visually for swarm response at various times. Experiments were performed at least two times with three replicates per treatment and analyzed independently.

### Microcapillary chemotaxis assay

Microcapillary chemotaxis assays were performed essentially as described (Adler 1973). Strains were grown overnight with and without 5 mM cAMP as for the motility assay. Fresh cultures were started in the same media and incubated until an O.D. 540 nm of 0.2–0.4 was reached. Cultures were washed twice in chemotaxis wash buffer and resuspended in chemotaxis buffer at approximately  $5.0 \times 10^6$  CFU ml<sup>-1</sup>. Microcapillaries containing various concentrations of synthetic root exudate medium (described above), individual reduced carbon compounds, or SDW were inserted into chemotaxis apparatus (described in Adler 1973) containing 150 µl of the bacterial suspensions and incubated at 28°C for 40 min. CFU in microcapillaries were determined by spiral plating onto LB agar containing appropriate antibiotics. Mean log<sub>10</sub> CFU with standard deviation was calculated for each treatment. All experiments were performed twice with three replicates per treatment and experiments analyzed independently.

### Root colonization assays

For whole-root colonization assays strains 501R3 and M59 were grown overnight at 250 rpm in LB broth containing appropriate antibiotics. Cucumber seeds treated with strains 501R3, M59, or no bacteria in a gel-

atin formulation (Roberts et al. 2005) were sown in potting mix (Redi-Earth, Scotts Horticultural Products, Marysville, OH, USA) or a natural Hatborough loamy sand soil (pH 5.9, 3.04% carbon) in 6.5-cm diameter × 25 cm deep pots and incubated in a growth chamber at 22°C with a 12 h photoperiod. Plants were removed at sampling time and the roots excised by cutting the plants at the soil line. The root and attached planting medium were placed in SDW, sonicated 5 min (Branson Ultrasonic Corp., Model 8210, Danbury, CT, USA), and CFU (root system)<sup>-1</sup> determined by spiral plating onto LB agar containing appropriate antibiotics (Roberts et al. 1997). Root system fresh weight was determined for each sample. The experiment was performed two times with each planting medium with six replicates per treatment. Mean log<sub>10</sub> CFU (g fresh weight root tissue)<sup>-1</sup> was determined and compared using Least Squares Means in proc GLM (SAS Institute, Cary, NC, USA). Experiments were analyzed independently.

For experiments to determine the distribution of populations of *Enterobacter cloacae* along roots, individual cucumber seeds treated with strains 501R3 or M59 in the gelatin formulation were sown in potting mix in deep pots as above. Seeds treated with gelatin only were used as controls. At sampling time 2-cm sections of root were placed in SDW, sonicated, and populations of 501R3 and M59 determined as above. The experiment was performed two times with six replicates per treatment. Mean log<sub>10</sub> CFU (2-cm root)<sup>-1</sup> was determined and compared using Least Squares Means in proc GLM. Experiments were analyzed independently.

### Biochemical restoration of seedling colonization

Strains were grown overnight at 250 rpm in LB broth containing appropriate antibiotics, washed twice with SDW, resuspended in SDW, and applied at approximately  $7.70 \log_{10}$  CFU seed<sup>-1</sup> to cucumber seeds in 4 ml potting mix in 14 ml snap-cap tubes. The potting mix had been equilibrated with 1 ml SDW or 5 mM cAMP in SDW. SDW (500 µl) and cAMP (500 µl, 5 mM) were added to appropriate treatments 74 and 244 h after application of bacteria. Populations of strains 501R3 and M59 were determined at various times by spiral plating as previously described (Roberts et al. 1992). The experiment was performed four times with six replicates per treatment. Mean log<sub>10</sub> CFU seedling<sup>-1</sup> was determined and compared using least squares means in proc GLM. Experiments were analyzed independently.

Control seedling colonization assays were conducted as above except strain M59 was applied at approximately

7.70 log<sub>10</sub> CFU seed<sup>-1</sup> to cucumber seeds in potting mix that had been equilibrated with 1 ml SDW; 5 mM CaNO<sub>3</sub>; or 5 mM KPO<sub>4</sub>, pH 7.0. SDW, 5 mM CaNO<sub>3</sub>, or 5 mM KPO<sub>4</sub>, pH 7.0 (500 µl) were added 74 h and 244 h after application of bacteria to appropriate treatments. The experiment was performed two times with six replicates per treatment. Mean log<sub>10</sub> CFU seedling<sup>-1</sup> was determined and compared using Least Squares Means in proc GLM. Experiments were analyzed independently.

## Results

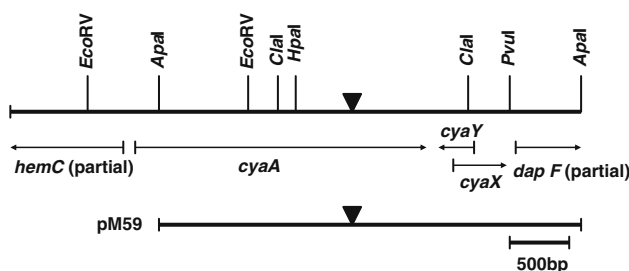
### Molecular and biochemical characterization of *Enterobacter cloacae* M59

*Enterobacter cloacae* strain M59 was selected for further study after the initial screening of a library of mini-Tn5 Km mutants of strain 501R3 for mutants reduced in seed colonization (Roberts et al. 1996). Southern hybridization analysis confirmed the presence of a single mini-Tn5 Km insertion in the genome of strain M59 (Roberts et al. 1996). Subsequent subcloning of the region of the M59 genome containing the mini-Tn5 Km insertion yielded plasmid pM59 (Fig. 1). Sequencing outward from mini-Tn5 Km in plasmid pM59, using primers specific for the ends of mini-Tn5 Km, indicated that this transposon was inserted in a region of the strain M59 genome with a high degree of sequence similarity to *cyaA* (Keseler et al. 2005). Plasmid pM59 contains only the downstream terminal portion of the *cyaA* gene, no promoter or ribosomal binding sites were identified (Fig. 1). Additional upstream sequence in the *Enterobacter cloacae* genome containing the

remaining portion of *cyaA* was obtained by directional walking. Comparison of the complete *cyaA* sequence from *Enterobacter cloacae* with the *cyaA* sequence from *E. coli* indicated 85.0% DNA sequence identity and 91.2% amino acid sequence similarity (data not shown). An open reading frame sufficient to encode a polypeptide of 848 amino acids was detected (data not shown). The unusual translation initiation codon TTG included within the 18-nucleotide sequence CAG-GCGATACGTCTTGTA, which is highly conserved in the Enterobacteriaceae (Danchin 1993), was identified. Consensus -10 and -35 sites for promoter P1 were identified extending from residues -338 to -333 and -361 to -356, respectively, relative to the predicted translation initiation site. Consensus -10 and -35 sites for promoter P2 were identified extending from residues -154 to -149 and -177 to -172, respectively, relative to the predicted translation initiation site. A ribosome-binding site was identified extending from residues -17 to -7 relative to the predicted translation initiation site.

Analysis of sequence upstream of *cyaA* indicated the presence of a region of DNA with a high degree of sequence similarity to *hemC* (Fig. 1). This gene encodes hydroxymethylbilane synthase (Keseler et al. 2005). Analysis of sequence downstream of *cyaA* indicated the presence of regions of DNA with high degrees of sequence similarity to *cyaY*, *cyaX*, and *dapF* (Fig. 1). These genes encode an iron-binding frataxin homolog, a hypothetical protein, and diaminopimilate epimerase, respectively (Keseler et al. 2005). The *hemC*, *cyaA*, *cyaY*, *cyaX*, and *dapF* genes have identical organization and direction of transcription in *Enterobacter cloacae* and *E. coli* K12 (Keseler et al. 2005). A GeneContext analysis revealed that this gene organization is also identical in *E. coli* strains O157:H7, O157:H7 EDL 933, and W3110, but differs from that of *E. coli* strains CFT073 and UTI89 and from *Erwinia caratovora* subsp. *atroseptica* SCRI1043. This gene organization is clearly not conserved in the Enterobacteriaceae as postulated earlier (Danchin 1993).

Results from Southern hybridization experiments were consistent with *Enterobacter cloacae* containing a single copy of *cyaA* in its genome. Genomic DNA preparations from strains 501R3 and M59, digested with the restriction endonucleases *PvuI*, *FspI*, *ClaI*, and *HpaI*, were hybridized to *cyaA* and mini-Tn5 Km probes. As determined by analysis of the 501R3 *cyaA* sequence, *PvuI*, *FspI*, *ClaI*, and *HpaI* cut within *cyaA* 0, 0, 1, and 1 time, respectively. *FspI* cuts within mini-Tn5 Km once while *PvuI*, *ClaI*, and *HpaI* do not cut within this minitransposon. Single bands were detected in genomic preparations from strain 501R3 digested



**Fig. 1** Physical maps of the portion of the *Enterobacter cloacae* M59 genome containing *cyaA* and of plasmid pM59. The location of certain restriction endonuclease cleavage sites are indicated. The location and direction of transcription of genes is indicated by the horizontal arrows. The downward pointing arrow indicates the mini-Tn5 Km insertion site at position 1,765 of *cyaA*. Partial indicates that only a portion of the sequence of this gene was obtained. The nucleotide sequence and sequence of the translated proteins have been deposited in GenBank under accession number AY765258

with *PvuI* and *FspI* after hybridization with the *cyaA* probe while two bands were detected with preparations digested with *ClaI* and *HpaI* (data not shown). A single band was also detected in genomic preparations from strain M59 digested with *PvuI* after hybridization with the *cyaA* probe. Two bands were detected in genomic preparations from strain M59 when digested with *FspI* due to the additional *FspI* restriction site located in mini-Tn5 Km. As with genomic preparations from strain 501R3, two bands were detected in M59 preparations digested with *ClaI* and *HpaI* after hybridization with the *cyaA* probe since there are no *ClaI* or *HpaI* located in mini-Tn5 Km (data not shown). The mini-Tn5 Km probe did not hybridize to genomic preparations from strain 501R3. Single bands were detected in genomic preparations from strain M59 digested with *PvuI*, *ClaI* and *HpaI* after hybridization with the mini-Tn5 Km probe while two bands were detected with preparations digested with *FspI* (data not shown). This is the expected profile for *Enterobacter cloacae* containing a single copy of *cyaA* in the genome.

The transposon insertion in *cyaA* in strain M59 resulted in dramatically reduced levels of cAMP relative to the wild-type strain. cAMP levels in strain M59 were approximately 2% of those in strain 501R3. Lysate from cultures of strain 501R3 grown at 28°C contained  $0.166 \pm 0.076$  pMole cAMP ( $\mu\text{g protein}^{-1}$ ) while lysate from strain M59 contained  $0.0029 \pm 0.0025$  pMole cAMP ( $\mu\text{g protein}^{-1}$ ). Lysate from cultures of strain 501R3 grown at 37°C contained  $0.05 \pm 0.03$  pMole cAMP ( $\mu\text{g protein}^{-1}$ ) while lysate from strain M59 contained  $0.001 \pm 0.0005$  pMole cAMP ( $\mu\text{g protein}^{-1}$ ).

The phosphomycin resistance profile of strain M59 grown with and without cAMP is consistent with that of *E. coli cyaA* mutants (Sakamoto et al. 2003). Phosphomycin is transported into *E. coli* cells via the glycerol-3-phosphate transporter whose production is dependent on the presence of cAMP (Tsuruoka and Yamada 1975; Alper and Ames 1978). The minimal concentration of phosphomycin tested that was inhibitory to strains 501R3 and M59 in the absence of cAMP was  $1,500 \mu\text{g ml}^{-1}$  and  $>6,000 \mu\text{g ml}^{-1}$ , respectively. The presence of 1 mM cAMP increased sensitivity to phosphomycin with both strains. The minimal concentration of phosphomycin tested that was inhibitory in the presence of 1 mM cAMP was  $1,000 \mu\text{g ml}^{-1}$  for 501R3 and  $2,000 \mu\text{g ml}^{-1}$  for strain M59. Similar results were obtained in a second experiment.

#### Environmental stress assays

*Enterobacter cloacae* M59 was more resistant to heat stress than the wild-type strain, strain 501R3.  $\text{Log}_{10}$

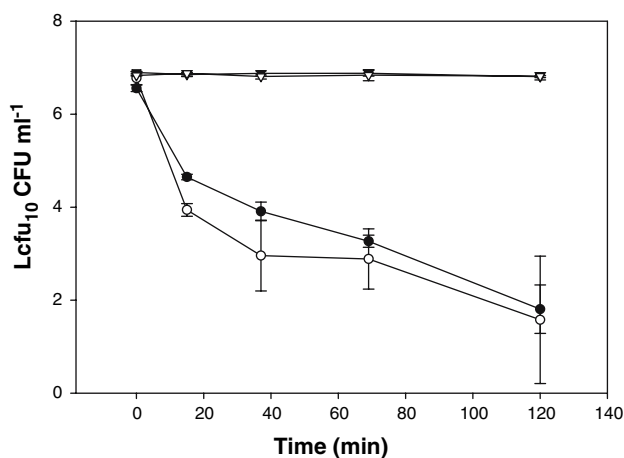
CFU for strains M59 and 501R3 after 0, 15, 30, 45, 60, 75, and 90 min incubation at 50°C were  $8.98 \pm 0.07$  and  $9.15 \pm 0.10$ ,  $8.33 \pm 0.02$  and  $8.28 \pm 0.11$ ,  $7.32 \pm 0.27$  and  $5.84 \pm 0.23$ ,  $6.53 \pm 0.04$  and  $4.87 \pm 0.23$ ,  $5.88 \pm 0.12$  and  $4.52 \pm 0.36$ ,  $5.21 \pm 0.11$  and  $2.31 \pm 2.00$ , and  $4.43 \pm 0.12$  and  $2.21 \pm 1.91$ , respectively. Similar results were obtained in a second experiment (data not shown).

Strain M59 was slightly less acid resistant than strain 501R3 (Fig. 2). In three of four experiments populations of strain 501R3 were significantly greater than those of strain M59 after 15–20 min incubation at pH 2.5 and trended higher than those of strain M59 for the remainder of the experiment. In one of four experiments populations of strain 501R3 were equal to, or slightly but significantly less, than those of strain M59 at all time points.

Strains M59 and 501R3 were similar in growth on all high-osmotic-strength media tested. Strains M59 and 501R3 grew equally well on N agar supplemented with 1.0 M and 2.0 M sorbitol, 1.0 and 2.0 M glucose, 0.5 and 1.0 M NaCl, and 0.5 and 1.0 M KCl. Neither strain grew on N agar supplemented with 3.0 M sorbitol, 3.0 M glucose, 1.5 M NaCl, or 1.5 M KCl. Similar results were obtained in both of the additional experiments.

#### Motility

*Enterobacter cloacae* strain M59 was motile. Evidence for motility was obtained microscopically and in tube assays using semisolid agar (Kreig and Gerhardt 1981)



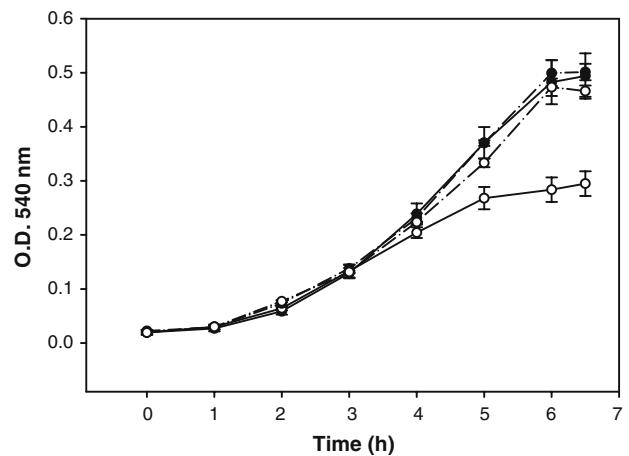
**Fig. 2** Acid resistance by *Enterobacter cloacae* strains 501R3 and M59. Acid resistance was determined by incubating strains 501R3 (closed circles) and M59 (open circles) in EG medium, pH 2.5, followed by dilution-plating. Strains 501R3 (closed triangles) and M59 (open triangles) were incubated in EG medium, pH 7.0, as a control. Error bars represent one standard deviation from the mean. Treatments were replicated three times

in repeated experiments (data not shown). Additionally, strain M59 was motile on swarm agar and in microcapillary chemotaxis assays (Tables 2, 3).

#### *In vitro* growth and chemotaxis on reduced carbon compounds in root exudate

*Enterobacter cloacae* M59 was significantly reduced in growth on all but one of the predominant carbohydrates, amino acids, and organic acids detected in cucumber root exudate when these compounds were supplied individually (Table 2). Similar results were obtained in two additional experiments (data not shown). Relative growth of *Enterobacter cloacae* strains M59 and 501R3 was also determined on synthetic cucumber root exudate supplied at various final concentrations. Strain M59 was reduced in growth relative to strain 501R3 on this synthetic cucumber root exudate medium when reduced carbon was supplied at 0.05% (Fig. 3) and 0.1% in M56 basal salts medium. Strain M59 was not significantly reduced in growth relative to strain 501R3 when this synthetic root exudate was supplied at 0.5% (data not shown). Similar results were obtained in four additional experiments (data not shown). There was no significant growth by either strain on synthetic root exudate when supplied at 0.01% in preliminary experiments (data not shown). The differential impact of the mutation in *cyaA* in strain M59 under low versus high concentrations of synthetic root exudate was possibly due to the role of cAMP in expression of ABC transport systems by enteric bacteria which function in scavenging nutrients present in low concentrations (Ferenci 1999).

Strain M59 was also reduced relative to strain 501R3 in swarm response to all compounds tested individually on swarm agar plates with the exception of arabinose, rhamnose, histidine, and serine (Table 2). Neither strain was responsive toward these four compounds on



**Fig. 3** Growth by *Enterobacter cloacae* strains 501R3 and M59 on 0.05% synthetic cucumber root exudate in the presence and absence of exogenous 1 mM cAMP. Open circles strain M59; closed circles strain 501R3; solid line no exogenous cAMP; dashed line, 1 mM cAMP present in growth medium. Mean optical density with standard deviation over time from a single experiment is shown. Treatments were replicated three times

swarm agar plates. Similar results were obtained in at least two additional experiments with each individual compound (data not shown). The swarm response was always only in the direction of the chemical source. The microcapillary chemotaxis assay of Adler (1973) was used to confirm that strain M59 was capable of limited chemotaxis toward some compounds detected in cucumber root exudates. Log<sub>10</sub> CFU of strains 501R3 and M59 in microcapillaries originally containing 0.1 mM glucose, 1 mM citrate, and SDW were  $6.60 \pm 0.19$  and  $6.06 \pm 0.16$ ,  $5.92 \pm 0.15$  and  $4.97 \pm 0.07$ , and  $4.99 \pm 0.25$  and  $4.91 \pm 0.05$ , respectively. In this experiment strain M59 was capable of chemotaxis toward 0.1 mM glucose, but at a reduced level relative to strain 501R3, and no longer capable of chemotaxis toward 0.1 mM citrate. Similar results were obtained in a second experiment (data not shown).

**Table 3** Chemotaxis by *Enterobacter cloacae* strains to synthetic cucumber root exudate

Chemoattractant	Log <sub>10</sub> CFU microcapillary <sup>-1</sup>			
	501R3	501R3 + cAMP	M59	M59 + cAMP
0.4% exudate	$6.48 \pm 0.30$	$6.59 \pm 0.21$	$5.08 \pm 0.11$	$6.41 \pm 0.06$
0.01% exudate	$6.21 \pm 0.32$	ND	$4.83 \pm 0.21$	ND
0.0004% exudate	$6.24 \pm 0.22$	$6.07 \pm 0.18$	$4.87 \pm 0.06$	$6.17 \pm 0.23$
SDW	$4.13 \pm 0.18$	$3.87 \pm 0.31$	$4.26 \pm 0.05$	$4.51 \pm 0.15$

Strains were grown in M56 salts plus 0.1% glycerol with and without 5 mM cAMP. The chemotaxis apparatus (Adler 1973) contained 150 µl of a bacterial suspension (approximately  $5 \times 10^6$  CFU ml<sup>-1</sup>). Microcapillaries containing chemoattractant were incubated in the apparatus at 28°C for 40 min prior to determining bacterial populations that had migrated into these microcapillary tubes

ND not determined, SDW sterile distilled water

There was substantial migration by strain 501R3 toward the synthetic root exudate on swarm agar with the leading edge of the swarm front of strain 501R3 located  $12.7 \pm 0.5$  mm from the point of application of bacteria when 10  $\mu$ l of 0.4% synthetic cucumber root exudate was used as chemoattractant. There was no detectable swarm response to this reduced carbon mixture by strain M59. Similar results were obtained in a second experiment (data not shown). In all experiments the swarm response was only in the direction of the chemical source. Chemotaxis by strain M59 to 0.4, 0.01, and 0.0004% synthetic cucumber root exudate, as determined by measuring bacterial populations in microcapillary tubes containing these mixtures, was significantly lower than that by strain 501R3 in microcapillary assays (Table 3). Chemotaxis by strain M59 using 0.01 and 0.004% synthetic root exudate as chemoattractant was marginally greater than the SDW control. Similar results were obtained in a second experiment (data not shown).

#### Colonization

In initial studies *Enterobacter cloacae* M59 was reduced in growth on germinating cucumber seeds relative to strain 501R3 after 48 h when applied at low initial population levels (Roberts et al. 1996). In the current study it was determined that *Enterobacter cloacae* M59 was significantly impaired in colonization of cucumber roots relative to *Enterobacter cloacae* 501R3. Populations of strains 501R3 and M59 were  $5.80 \log_{10}$  CFU (g root) $^{-1}$  and  $1.54 \log_{10}$  CFU (g root) $^{-1}$  ( $P < 0.0001$ ) at 21 days, and  $4.65 \log_{10}$  CFU (g root) $^{-1}$  and  $0.57 \log_{10}$  CFU (g root) $^{-1}$  ( $P < 0.0001$ ) at 42 days, respectively, in one experiment conducted with non-segmented root systems from cucumber plants grown in natural soil. In a second experiment conducted in natural soil, populations of 501R3 and M59 were similar at 21 days ( $P = 0.21$ ) and significantly different at 42 days ( $P = 0.0013$ ) (data not shown). Populations of strain 501R3 were at least 700-fold greater than those of strain M59 at one or more time points in both of these experiments. Populations of strains 501R3 and M59 were  $5.07 \log_{10}$  CFU (g root) $^{-1}$  and  $0.66 \log_{10}$  CFU (g root) $^{-1}$  ( $P < 0.0001$ ) at 25 days and  $5.73 \log_{10}$  CFU (g root) $^{-1}$  and  $2.55 \log_{10}$  CFU (g root) $^{-1}$  ( $P = 0.0009$ ) at 40 days, respectively, in studies conducted with non-segmented root systems from cucumber plants grown in potting mix. In a second experiment conducted in potting mix, populations of 501R3 and M59 were significantly different at 21 days ( $P = 0.004$ ) and similar at 42 days ( $P = 0.15$ ) (data not shown). Populations of strain 501R3 were only sixfold greater than those of

strain M59 in the second experiment conducted in potting mix. Initial populations in these experiments with non-segmented root systems were approximately  $7.50 \log_{10}$  CFU seed $^{-1}$  for each strain.

The distribution of *Enterobacter cloacae* strains along cucumber roots was determined in a second set of experiments where the root system from cucumber plants grown in potting mix was cut into 2-cm-long segments. In these experiments, populations of *Enterobacter cloacae* 501R3 decreased in the rhizosphere with increasing distance from the soil line at 27 and 47 days after sowing bacterized seed but were extensively distributed along cucumber roots (Table 4). Populations of *Enterobacter cloacae* M59 were only fivefold less than those of strain 501R3 on the uppermost 2-cm root segment. However, populations of strain M59 decreased much more rapidly than those of strain 501R3 with increasing distance from the soil line (Table 4). Similar results were obtained in a second experiment (data not shown). Initial populations in these experiments were approximately  $8.00 \log_{10}$  CFU seed $^{-1}$  for each strain.

#### Biochemical restoration of *in vitro* growth, chemotaxis, and seedling colonization

Addition of 1 mM cAMP to M56 salts broth amended with 0.05% synthetic root exudate resulted in a significant

**Table 4** Colonization of cucumber roots by *Enterobacter cloacae* strains

Day <sup>a</sup>	Root segment (cm) <sup>b</sup>	Log <sub>10</sub> CFU segment $^{-1}$		
		501R3	M59	Pr > T
27	0–2	6.61	5.93	0.41
	2–4	5.34	4.47	0.29
	4–6	4.94	4.05	0.28
	6–8	4.77	2.81	0.02
	8–10	4.79	2.75	0.02
	10–12	5.03	2.62	0.004
	12–14	4.67	1.42	0.0001
	14–16	3.39	0.73	0.003
	16–18	2.16	BDL <sup>c</sup>	0.18
47	0–2	6.82	6.02	0.33
	2–4	5.40	3.83	0.06
	4–6	5.17	2.06	0.0002
	6–8	4.66	BDL	0.0001
	8–10	3.50	BDL	0.0001
	10–12	2.17	0.69	0.07
	12–14	3.09	BDL	0.0003
	14–16	2.28	0.70	0.06

Results from a single experiment are presented. Initial populations were approximately  $8.00 \log_{10}$  CFU seed $^{-1}$  for each strain

<sup>a</sup> Days after sowing bacterized cucumber seed

<sup>b</sup> Distance down the root from the soil line

<sup>c</sup> BDL below detectable limit

increase in in vitro growth by strain M59 (Fig. 3). Growth by strain M59 in the presence of exogenous cAMP was significantly greater than that by M59 in the absence of exogenous cAMP and similar to that of strain 501R3 with and without addition of exogenous cAMP. Similar results were obtained in two additional experiments (data not shown). Swarm response by strain M59 (with exogenous cAMP) toward 0.4% synthetic root exudates was restored to levels associated with strain 501R3 with and without exogenous cAMP on swarm agar plates. The distance of the leading edge of the swarm front from the point of application of bacterial treatments was  $11.7 \pm 0.5$ ,  $12.7 \pm 0.5$ ,  $0.0 \pm 0.0$ , and  $11.3 \pm 0.5$  for strain 501R3 without exogenous cAMP, strain 501R3 with exogenous cAMP, strain M59 without exogenous cAMP, and strain M59 with exogenous cAMP, respectively. Likewise, addition of exogenous cAMP restored chemotaxis in microcapillary assays by strain M59 toward synthetic root exudate to levels similar to those of strain 501R3 in the presence and absence of cAMP (Table 3). Similar results were obtained in repeated experiments (data not shown). Interestingly, higher concentrations of exogenous cAMP (3 mM) were inhibitory to in vitro growth and the swarm response by both strains 501R3 and M59 in the presence of synthetic root exudate medium (data not shown). The inhibitory effects of higher concentrations of cAMP on growth and swarm response were possibly due to negative regulation exerted by excess levels of the cAMP–CRP complex on the P2 promoter of *cyaA* (Aiba 1985).

Addition of 5 mM cAMP to potting mix allowed strain M59 to maintain populations on cucumber seedlings longer than when no cAMP was added (Fig. 4a). Populations of strain M59 decreased more rapidly than populations of strain 501R3 on cucumber seedlings with populations of strain M59 being significantly lower ( $P \leq 0.05$ ) than those of strain 501R3 at 240 and 312 h after application of bacteria to cucumber seed. cAMP was added to certain treatments 74 and 242 h after application of bacteria to seeds. Populations of M59 in the presence of exogenous cAMP were slightly greater than those of strain M59 where no cAMP was added at 144 h. Populations of M59 where exogenous cAMP was added were significantly greater ( $P \leq 0.05$ ) than those of strain M59 where no cAMP was added at 312 h and similar to those of strain 501R3 in the absence of cAMP. Populations of strain 501R3 in the presence of cAMP also trended higher than those of 501R3 in the absence of cAMP at 144 and 312 h. Similar results were obtained in three other experiments (data not shown). This effect of cAMP on populations of strain M59 on cucumber seedlings was not due to

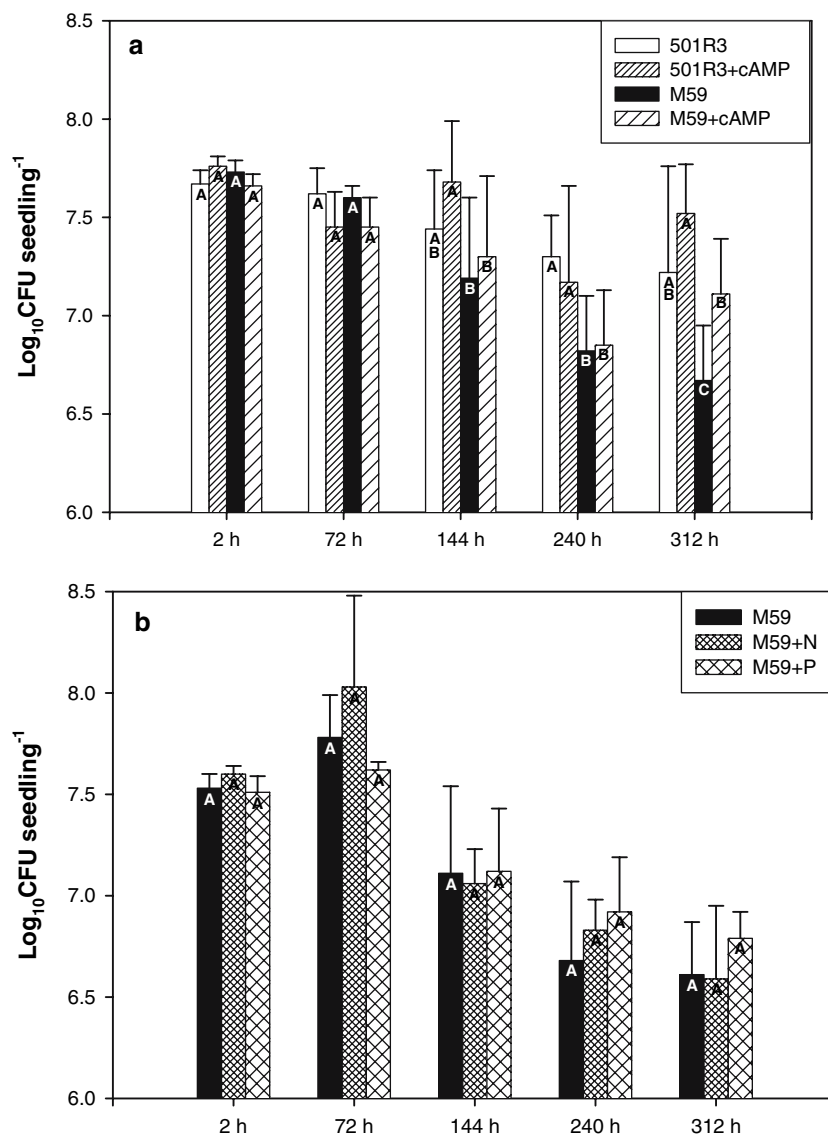
this molecule serving as a source of carbon, nitrogen, or phosphorous. *Enterobacter cloacae* strains 501R3 and M59 did not grow on cAMP in vitro when this molecule was supplied as the sole source of carbon, nitrogen, or phosphorous (data not shown). In addition, populations of strain M59 on cucumber seedlings were similar ( $P > 0.19$ ) at all sampling times in all treatments when SDW, 5 mM  $\text{CaNO}_3$ , or 5 mM  $\text{KPO}_4$ , pH 7.0, were added (Fig. 4b). Similar results were obtained in a second experiment (data not shown).

## Discussion

cAMP, whose synthesis is catalyzed by adenylate cyclase, plays a universal role in the regulation of gene expression at the transcriptional level and in the integration of metabolic functions (Danchin 1993). We report for the first time that cAMP and *cyaA*, the gene that encodes adenylate cyclase, have an important role in root colonization by plant-beneficial bacteria. The following genetic and biochemical data demonstrate this: *E. cloacae* M59 was reduced in cucumber root colonization and contained a single mini-Tn5 Km insertion in a region of the *Enterobacter cloacae* genome with very high DNA and amino acid sequence similarity to *cyaA* from *E. coli* (data not shown). *Enterobacter cloacae* M59 had dramatically lower adenylate cyclase activity than strain 501R3, with cellular cAMP levels approximately 2% those detected in strain 501R3. As with *E. coli cyaA* mutants, strain M59 was more resistant to heat stress and the antibiotic phosphomycin than the wild-type strain, strain 501R3 (Tsuruoka and Yamada 1975; Alper and Ames 1978; Delaney 1990; Lee-Rivera and Gomez-Eichelmann 1994). Finally, addition of exogenous cAMP improved colonization of cucumber rhizosphere by strain M59 (Fig. 4) indicating that the strain M59 colonization phenotype was due to the mutation in *cyaA* and not to other physically linked genes.

Although *Enterobacter cloacae* M59 clearly contained a single transposon insertion in its genome within *cyaA*, M59 differs from other enteric *cyaA* mutants in certain phenotypes. (1) Strain M59 was only slightly less acid resistant than strain 501R3 (Fig. 2) whereas *cyaA* and *crp* mutants of *E. coli* have been shown to be present in populations that are orders of magnitude lower than those of the wildtype after 2 h incubation at pH 2.5 (Castanie-Cornet et al. 1999). (2) We did not detect differences in growth between strains M59 and 501R3 on high-osmotic-strength media. (3) Strain M59 was motile and capable of limited chemotaxis, unlike most other enteric *cyaA*

**Fig. 4** Colonization of cucumber seedlings by *Enterobacter cloacae* strains 501R3 and M59 in the presence and absence of various amendments. **a** +cAMP indicates 5 mM cAMP (1 ml) was incorporated in the potting mix prior to planting and 500  $\mu$ l 5 mM cAMP added at 74 and 244 h after planting. Other treatments had similar volumes of sterile distilled water added at these times. **b** + N and + P indicate 1 ml 5 mM  $\text{CaCO}_3$  and 5 mM  $\text{KPO}_4$ , respectively, added prior to planting and 500  $\mu$ l added 74 and 244 h after planting. The other treatments had similar volumes of sterile distilled water added at these times. Treatment values are the mean of six replicates. Treatment values with similar letters are not significantly different ( $P \leq 0.05$ ) at that time. Results are from a single experiment. Error bars represent one standard deviation from the mean



mutants, which typically are nonmotile. *cyaA* is involved in motility and chemotaxis in enteric bacteria due to the requirement of cAMP for biosynthesis of flagellar proteins and certain chemoreceptors that detect carbon sources (Rephaeli and Saier 1976; Vogler and Lengeler 1987). (4) Strain M59 was capable of limited growth on phosphotransferase sugars (e.g. cellibiose, glucose, mannose), Class I (e.g. raffinose, data not shown), and Class II (e.g. galactose, malate, rhamnose, xylose) compounds (Table 2). *E. coli cyaA* mutants are deficient in growth on many of these compounds (Postma et al. 1996).

These phenotype differences between strain M59 and other enteric *cyaA* mutants may be due to the location of the mini-Tn5 Km insertion within *cyaA* in M59. The mini-Tn5 Km insertion at position 1,765 (data not shown) in the nucleotide sequence of *cyaA* likely

affects production of the C-terminal region of adenylate cyclase in strain M59 (Fig. 1). This insertion is at residue 588 (total of 848 residues) when considering the amino acid sequence. The catalytic domain occupies the N-terminal portion of adenylate cyclase, with the limit of the catalytic domain slightly upstream of residue 414 (Trotot et al. 1996). The C-terminal domain of adenylate cyclase in *E. coli* has been shown to be non-essential for catalytic activity and is postulated to be involved in regulation (Crasnier and Danchin 1990; Crasnier et al. 1994; Saier et al. 1996). Certain *E. coli* mutants, with mutations in this region, produce an adenylate cyclase that synthesizes cAMP at reduced rates (Crasnier et al. 1994). Whether the phenotype differences between strain M59 and other enteric *cyaA* mutants are due to regulation per se or a truncated adenylate cyclase, and resultant low intracellular levels

of cAMP, are hypotheses that await further experimentation and confirmation.

The plant spermosphere and rhizosphere are nutritionally complex environments that vary spatially and temporally (Curl and Truelove 1986). For effective applications of plant-beneficial bacteria these microbes must adapt to these different environments to obtain carbon and energy for growth and production of metabolites involved in disease suppression. Supporting this, beneficial pseudomonads have been shown to adapt to plant rhizosphere through differential expression of genes, including genes with homology to genes involved in transport and metabolism of reduced carbon compounds (Rainey 1999). Also, certain nutritional traits vary greatly in importance in the spermosphere environments of different seed types (Roberts et al. 1996, 1999, 2000). Finally, root colonization experiments presented here suggest that the impact of the mutation in *cyaA* on cucumber root colonization by *Enterobacter cloacae* varies with planting medium. *Enterobacter cloacae* M59 was dramatically reduced in population in natural soil relative to *Enterobacter cloacae* 501R3 in two of two experiments but only slightly reduced (approximately fivefold) relative to *Enterobacter cloacae* 501R3 in potting mix in three of four experiments (entire root and segmented root experiments collectively). It is possible that competition for available reduced carbon varied between these planting media due to differences in the indigenous microbial community.

Understanding how beneficial bacteria respond to different nutritional environments and regulate expression of genes involved in acquisition and metabolism of reduced carbon nutrients remains a significant challenge. We identify here the importance of cAMP, and by extension, cAMP–CRP regulated gene expression in the colonization of cucumber roots by the plant-beneficial bacterium *Enterobacter cloacae*. It is likely that the role of cAMP in colonization has to do, at least in part, with regulation of genes involved in acquisition and metabolism of reduced carbon compounds in the rhizosphere. Strain M59 was substantially impacted in traits involved in the acquisition of reduced carbon; being reduced in chemotaxis and growth on almost all reduced carbon compounds detected in cucumber root exudates (Tables 2, 3; Fig. 3). In addition to data presented here, previous work has shown that almost all genes in enteric bacteria that encode enzymes involved in transport and metabolism of reduced carbon compounds are under cAMP–CRP control (Saier et al. 1996). It should be noted that cAMP-independent catabolite repression pathways have been identified in enteric bacteria (Saier 1996).

It is possible that the importance of cAMP in the association of *Enterobacter cloacae* with cucumber roots also has to do with traits other than the acquisition and utilization of reduced carbon. Transcriptome analysis of CRP-dependent catabolite control of gene expression in *E. coli* identified a variety of stress-related genes that are regulated in response to the presence of CRP (Gosset et al. 2004). In addition, more than half of all  $\sigma^S$ -controlled genes contain potential cAMP–CRP binding sites in adjacent upstream regions (Weber et al. 2005). The general stress sigma factor,  $\sigma^S$ , is strongly induced when *E. coli* cells are exposed to starvation, osmotic, nonoptimal temperature, and acidic stress environments (Hengge-Aronis 2002). Acidic, osmotic, and starvation stress conditions have been observed, or can easily be envisioned, in the plant spermosphere and rhizosphere (Jones et al. 2004). Other previously unidentified traits directly or indirectly under cAMP control may also be involved in colonization of cucumber roots by *Enterobacter cloacae*. CRP has been shown to regulate transcription of other transcription factors such as Fur, MelR, RphH, BlgG, Fis, and PdhR, which could result in indirect effects of cAMP on expression of numerous genes (Kolb et al. 1993; Zheng et al. 2004; Zhang et al. 2005). Transcriptional profiling experiments identified 176 operons that were activated by CRP in *E. coli* and 16 operons that were repressed (Zheng et al. 2004). Analysis of these operons supports the hypothesis that CRP regulates the expression of a large number of genes not involved in the catabolism of reduced carbon compounds. Clearly further work needs to be performed to determine the environmental factors that influence cAMP–CRP gene regulation in plant spermosphere and rhizosphere.

**Acknowledgments** We thank Dr. Li Wei, Ernest Williams, and Ricky Brathwaite for assistance with certain experiments. We also thank Dr. Steve Rehner, USDA-ARS, Beltsville, MD, for assistance in sequencing *cyaA*.

## References

- Adler J (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J Gen Microbiol 74:77–91
- Aiba H (1985) Transcription of the *Escherichia coli* adenylate cyclase gene is negatively regulated by cAMP–cAMP receptor protein. J Biol Chem 260:3063–3070
- Aiba H, Kawaura R, Yamamoto E, Yamada H, Takegawa K, Mizuno T (1998) Isolation and characterization of high-osmolarity-sensitive mutants of fission yeast. J Bacteriol 180:5038–5043
- Alper MD, Ames BN (1978) Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive

- selection of *Salmonella typhimurium cya* and *crp* mutants. J Bacteriol 133:149–157
- Altschul SF, Madden TL, Schaffer AA, Zhang J, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402
- Bloemberg GV, Lugtenberg BJJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350
- Botsford JL, Harman JG (1992) Cyclic AMP in prokaryotes. Microbiol Rev 56:100–122
- Bull CT, Weller DM, Thomashow LS (1991) Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2–79. Phytopathology 81:954–959
- Bull CT, Duffy B, Voisard C, Défago G, Keel C, Haas D (2001) Characterization of spontaneous *gacS* and *gacA* regulatory mutants of *Pseudomonas fluorescens* biocontrol strain CHA0. Ant Leeuw 79:327–336
- Castanie-Cornet M-P, Penfound TA, Smith D, Elliot JF, Foster JW (1999) Control of acid resistance in *Escherichia coli*. J Bacteriol 181:3525–3535
- Chancey ST, Wood DW, Pierson EA, Pierson LS III (2002) Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30–84 in the wheat rhizosphere. Appl Environ Microbiol 68:3308–3314
- Chin-A-Wong TFC, Bloemberg GV, Mulders IHM, Dekkers LC, Lugtenberg BJJ (2000) Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Molec Plant-Microbe Interact 13:1340–1345
- Ciria R, Abreu-Goodger C, Morett E, Merino E (2004) GeConT: gene context analysis. Bioinformatics 20:2307–2308
- Crasnier M, Danchin A (1990) Characterization of *Escherichia coli* mutants with altered regulation. J Gen Microbiol 136:1825–1832
- Crasnier M, Dumay V, Danchin A (1994) The catalytic domain of *Escherichia coli* K-12 adenylate cyclase as revealed by deletion analysis of the *cya* gene. Mol Gen Genet 241:409–416
- Curl EA, Truelove B (1986) The rhizosphere, Springer, Berlin Heidelberg New York, pp 288
- Danchin A (1993) Phylogeny of adenylate cyclases. Adv Sec Mess Phosphoprot Res 27:109–162
- Dekkers LC, Bloemendaal CJP, de Weger LA, Wijffelman CA, Spaik HP, Lugtenberg BJJ (1998) A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. Mol Plant-Microbe Interact 11:45–56
- Delaney JM (1990) A *cya* deletion mutant of *Escherichia coli* develops thermotolerance but does not exhibit a heat-shock response. Genet Res, Camb 55:1–6
- de Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV, Vanderleyden J, De Mot R, Lugtenberg BJJ (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. Molec Plant-Microbe Interact 15:1173–1180
- Ferenci T (1999) Regulation by nutrient limitation. Curr Opin Microbiol 2:208–213
- Gosset G, Zhang Z, Nayyar S, Cuevas WA, Saier MH Jr (2004) Transcriptome analysis of Crp-dependent catabolite control of gene expression in *Escherichia coli*. J Bacteriol 186:3516–3524
- Gutierrez-Rios RM, Rosenblueth DA, Loza JA, Huerta AM, Glasner JD, Blattner FR, Collado-Vides J (2003) Regulatory network of *Escherichia coli*: consistency between literature knowledge and microarray profiles. Genome Res 13:2435–2443
- Hadari Y, Harman GE, Taylor AG, Horton JM (1983) Effects of pregermination of pea and cucumber seeds and of seed treatment with *Enterobacter cloacae* on rots caused by *Pythium* spp. Phytopathology 73:1322–1325
- Hawes MC, Smith LY, Howarth AJ (1988) *Agrobacterium tumefaciens* mutants deficient in chemotaxis to root exudates. Molec Plant-Microbe Interact 1:182–186
- Heeb S, Haas D (2001) Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. Molec Plant-Microbe Interact 12:1351–1363
- Hengge-Aronis R (2002) Signal transduction and regulatory mechanisms involved in control of the  $\sigma^s$  subunit of RNA polymerase in *Escherichia coli*. Microbiol Mol Biol Rev 66:373–395
- Jones DL, Hodge A, Kuzyakov Y (2004) Plant and mycorrhizal regulation of rhizodeposition. New Phytol 163:459–480
- Kaiser AD, Hogness DS (1960) The transformation of *Escherichia coli* with deoxyribonucleic acid isolated from the bacteriophage  $\lambda$ dg. J Molec Biol 2:392–415
- Keseler IM, Collado-Vides J, Gama-Castro S, Ingraham J, Paley S, Paulsen IT, Peralta-Gil M, Karp PD (2005) EcoCyc: a comprehensive database resource for *Escherichia coli*. Nuc Acids Res 33:D334–D337
- Kimura Y, Mishima Y, Nakano H, Takegawa K (2002) An adenylate cyclase, CyaA, of *Myxococcus xanthus* functions in signal transduction during osmotic stress. J Bacteriol 184:3578–3585
- Kolb A, Busby S, Buc H, Garges S, Adhya S (1993) Transcriptional regulation by cAMP and its receptor protein. Annu Rev Biochem 62:749–795
- Kreig NR, Gerhardt P (1981) Solid culture. In: Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (eds) Manual of methods for general bacteriology. American Society for Microbiology Press, Washington, pp 143–150
- Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJJ (2004) Rhizoremediation: a beneficial plant-microbe interaction. Molec Plant-Microbe Interact 17:6–15
- Lee-Rivera I, Gomez-Eichelmann MC (1994) *Escherichia coli* cells with mutations in the gene for adenylate cyclase (*cya*) exhibit a heat shock response. FEMS Microbiol Lett 121:35–38
- Lohrke SM, Dery PD, Li W, Reedy R, Kobayashi DY, Roberts DP (2002) Mutation in *rpiA* in *Enterobacter cloacae* decreases seed and root colonization and biocontrol of damping-off caused by *Pythium ultimum* on cucumber. Molec Plant-Microbe Interact 15:817–825
- Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 39:461–490
- Ma Z, Richard H, Foster JW (2003) pH-dependent modulation of cyclic AMP levels and GadW-dependent repression of RpoS affect synthesis of the GadX regulator and *Escherichia coli* acid resistance. J Bacteriol 185:6852–6859
- Martínez-Granero F, Capdevila S, Sanchez -Contreras M, Martín M, Rivilla R (2005) Two site-specific recombinases are implicated in phenotypic variation and competitive rhizosphere colonization in *Pseudomonas fluorescens*. Microbiol 151:975–983
- Mazzola M, Cook RJ, Thomashow LS, Weller DM, Pierson LS (1992) Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl Environ Microbiol 58:2616–2624
- Mishra RNS-P, Nair S, Sopory SK, Reddy MK (2002) Directional genome walking using PCR. Biotechniques 33:830–834

- Natsch A, Keel C, Pfrter HA, Haas D, Défago G (1994) Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. *Appl Environ Microbiol* 60:2553–2560
- Nelson EB (1988) Biological control of Pythium seed rot and pre-emergence damping-off of cotton with *Enterobacter cloacae* and *Erwinia herbicola* applied as seed treatments. *Plant Dis* 72:140–142
- Nguyen ND, Gottfert M, Singh M, Klingmüller W (1983) Nif-hybrids of *Enterobacter cloacae*: selection for *nif*-gene integration with *nif*-plasmids containing the Mu transposon. *Mol Gen Genet* 192:439–443
- Postma PW, Lengeler JS, Jacobson GR (1996) Phosphoenolpyruvate:carbohydrate phosphotransferase systems. In: Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (eds) *Escherichia coli* and *Salmonella*. Cellular and molecular biology, vol 1. American Society for Microbiology Press, Washington, pp 1149–1174
- Rainey PB (1999) Adaptation of *Pseudomonas fluorescens* to the plant rhizosphere. *Environ Microbiol* 1:243–257
- Rephaeli AW, Saier MH Jr (1976) Effects of *crp* mutations on adenosine 3',5'-monophosphate metabolism in *Salmonella typhimurium*. *J Bacteriol* 127:120–127
- Roberts DP, Sheets CJ, Hartung JS (1992) Evidence for proliferation of *Enterobacter cloacae* on carbohydrates in cucumber and pea spermosphere. *Can J Microbiol* 38:1128–1134
- Roberts DP, Marty AM, Dery PD, Yucel I, Hartung JS (1996) Amino acids as reduced carbon sources for *Enterobacter cloacae* during colonization of the spermospheres of crop plants. *Soil Biol Biochem* 28:1015–1020
- Roberts DP, Dery PD, Hebbar PK, Mao W, Lumsden RD (1997) Biological control of damping-off of cucumber caused by *Pythium ultimum* with a root-colonization-deficient strain of *Escherichia coli*. *J Phytopathol* 145:387–392
- Roberts DP, Dery PD, Yucel I, Buyer JS, Holtman MA, Kobayashi DY (1999) Role of *pfkA* and general carbohydrate catabolism in seed colonization by *Enterobacter cloacae*. *Appl Environ Microbiol* 65:2513–2519
- Roberts DP, Dery PD, Yucel I, Buyer JS (2000) Importance of *pfkA* for rapid growth of *Enterobacter cloacae* during colonization of crop seeds. *Appl Environ Microbiol* 66:87–91
- Roberts DP, Lohrke SM, Meyer SLF, Buyer JS, Bowers JH, Baker CJ, Li W, de Souza JT, Lewis JA, Chung S (2005) Biocontrol agents applied individually and in combination for suppression of soilborne diseases of cucumber. *Crop Prot* 24:141–155
- Saier MH Jr (1996) Cyclic AMP-independent catabolite repression bacteria. *FEMS Microbiol Lett* 138:97–103
- Saier MH Jr, Ramseier TM, Reizer J (1996) Regulation of carbon utilization. In: Neidhardt FC, Curtiss R III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (eds) *Escherichia coli* and *Salmonella*. Cellular and molecular biology, vol 1. American Society for Microbiology Press, Washington, pp 1325–1343
- Sakamoto Y, Furukawa S, Ogihara H, Yamasaki M (2003) Fosmidomycin resistance in adenylate cyclase deficient (*cya*) mutants of *Escherichia coli*. *Biosci Biotechnol Biochem* 67:2030–2033
- Sambrook J, Russell DW (2001) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Tang W-J, Gilman AG (1992) Adenylyl cyclases. *Cell* 70:869–872
- Thompson JE, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Res* 22:4673–4680
- Trotot P, Sismeiro O, Vivarjs C, Glaser P, Bresson-Roy A, Danchin A (1996) Comparative analysis of the *cya* locus in enterobacteria and related Gram-negative facultative anaerobes. *Biochimie* 78:277–287
- Tsuruoka T, Yamada Y (1975) Characterization of spontaneous fosfomycin (phosphonomycin)-resistant cells of *Escherichia coli* B in vitro. *J Antibiot* 28:906–911
- Vogel HJ, Bonner DM (1956) Acetylornithinase of *Escherichia coli*: Partial purification and some properties. *J Biol Chem* 218:97–106
- Vogler AP, Lengeler JW (1987) Indirect role of adenylate cyclase and cyclic AMP in chemotaxis to phosphotransferase system carbohydrates in *Escherichia coli* K-12. *J Bacteriol* 169:593–599
- Weber H, Polen T, Heuveling J, Wendisch VF, Hengge R (2005) Genome-wide analysis of the general stress response network in *Escherichia coli*:  $\sigma^s$ -dependent genes, promoters, and sigma factor selectivity. *J Bacteriol* 187:1591–1603
- Whistler CA, Corbell NA, Sarniguet A, Ream W, Loper JE (1998) The two-component regulators of GacS and GacA influence accumulation of the stationary-phase sigma factor  $\sigma^s$  and the stress response in *Pseudomonas fluorescens* Pf-5. *J Bacteriol* 180:6635–6641
- Zhang Z, Gosset G, Barabote R, Gonzalez CS, Cuevas WA, Saier MH Jr (2005) Functional interactions between the carbon and iron utilization regulators, Crp and Fur, in *Escherichia coli*. *J Bacteriol* 187:980–990
- Zheng D, Constantinidou C, Hobman JL, Minchin SD (2004) Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. *Nuc Acids Res* 32:5874–5893